

## CXCR4 AGONIST TREATMENT OF HEMATOPOIETIC CELLS

### FIELD OF THE INVENTION

In one aspect, the invention relates to therapeutic uses of chemokine receptor agonists, including peptide agonists of CXC chemokine receptor 4 (CXCR4) for use in the treatment of hematopoietic cells *in vitro* and *in vivo*.

### BACKGROUND OF THE INVENTION

Hematopoiesis consists of developmental cascades in which the hematopoietic stem cells generate lineage-committed cells and repeat the process of self-renewal. Hematopoietic stem cells are typically cells that have dual capacity for self-renewal and multilineage differentiation.

Cytokines are soluble proteins secreted by a variety of cells including monocytes or lymphocytes that regulate immune responses. Chemokines are a superfamily of chemoattractant proteins that may be classified into four groups, characterized by the nature of cysteine residues that are involved in disulfide bond formation. Chemokines regulate a variety of biological responses and they promote the recruitment of multiple lineages of leukocytes and lymphocytes to a body organ tissue. Chemokines may be classified into two families according to the relative position of the first two cysteine residues in the protein. In CC chemokines, which include beta chemokine the first two cysteines are adjacent to each other. In CXC chemokines, which include alpha chemokine, the first two cysteines are separated by one amino acid residue. Two minor subgroups contain only one of the two cysteines (C) or have three amino acids between the cysteines (CX<sub>3</sub>C). In humans, the genes of the CXC chemokines are clustered on chromosome 4 (with the exception of SDF-1 gene, which has been localized to chromosome 10) and those of the CC chemokines on chromosome 17.

The molecular targets for chemokines are cell surface receptors. One such receptor is CXC chemokine receptor 4 (CXCR4), which is a G-protein coupled seven transmembrane protein, and was previously called LESTR (Loetscher, M., Geiser, T., O'Reilly, T., Zwahlen, R., Baggionini, M., and Moser, B., (1994) *J. Biol. Chem.* 269, 232-237), HUMSTR (Federspiel, B., Duncan, A.M.V., Delaney, A., Schappert, K., Clark-Lewis, I., and Jirik, F.R. (1993) *Genomics* 16, 707-712) and Fusin (Feng, Y., Broeder, C.C., Kennedy, P.E., and Berger, E.A. (1996) *Science* 272, 872-877). CXCR4 is widely expressed on cells of hemopoietic origin, and is a major co-receptor with CD4<sup>+</sup> for human immunodeficiency virus 1 (HIV-1) (Feng, Y., Broeder, C.C., Kennedy, P.E., and Berger, E.A. (1996) *HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane G-protein-coupled receptor*, *Science* 272, 872-877).

Chemokines are thought to mediate their effect by binding to seven-transmembrane G protein-coupled receptors, and to attract leukocyte subsets to sites of inflammation (Baglionini *et al.* (1998) *Nature* 392: 565-568). Many of the chemokines have been shown to be constitutively expressed in lymphoid tissues, indicating that they may have a homeostatic function in regulating lymphocyte trafficking between and within lymphoid organs (Kim and Broxmeyer (1999) *J. Leuk. Biol.* 56: 6-15).

Stromal cell derived factor one (SDF-1) is a member of the CXC family of chemokines that has been found to be constitutively secreted from the bone marrow stroma (Tashiro, (1993) *Science* 261, 600-602). The human and mouse SDF-1 predicted protein sequences are approximately 92% identical. Stromal cell derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) and stromal cell derived factor-1 $\beta$  (SDF-1 $\beta$ ) are closely related (together referred to herein as SDF-1). The native amino acid sequences of SDF-1 $\alpha$  and SDF-1 $\beta$  are known, as are the genomic sequences encoding these proteins (see U.S. Patent No. 5,563,048 issued 8 October 1996, and U.S. Patent No. 5,756,084 issued 26 May 1998). Identification of genomic clones has shown that the alpha and

beta isoforms are a consequence of alternative splicing of a single gene. The alpha form is derived from exons 1-3 while the beta form contains an additional sequence from exon 4. The entire human gene is approximately 10 Kb. SDF-1 was initially characterized as a pre-B cell-stimulating factor and as  
5 a highly efficient chemotactic factor for T cells and monocytes (Bieul *et al.* (1996) *J. Exp. Med.* 184:1101-1110).

Biological effects of SDF-1 may be mediated by the chemokine receptor CXCR4 (also known as fusin or LESTR), which is expressed on  
10 mononuclear leukocytes including hematopoietic stem cells. SDF-1 is thought to be the natural ligand for CXCR4, and CXCR4 is thought to be the natural receptor for SDF-1 (Nagasawa *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 93:726-732). Genetic elimination of SDF-1 is associated with parinatal lethality, including abnormalities in cardiac development, B-cell  
15 lymphopoiesis, and bone marrow myelopoiesis (Nagasawa *et al.* (1996) *Nature* 382:635-637).

SDF-1 is functionally distinct from other chemokines in that it is reported to have a fundamental role in the trafficking, export and homing of  
20 bone marrow progenitor cells (Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Guierrez-Ramos, J.C., (1996) *J. Exp. Med.* 185, 111-120 and Nagasawa, T., Hirota, S., Tachibana, K., Takakura N., Nishikawa, S.-I., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T., (1996) *Nature* 382, 635-638). SDF-1 is also structurally distinct in that it has only about 22% amino acid  
25 sequence identity with other CXC chemokines (Bleul, C.C., Fuhrbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A., (1996) *J. Exp. Med.* 184, 1101-1109). SDF-1 appears to be produced constitutively by several cell types, and particularly high levels are found in bone-marrow stromal cells (Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H. Shinohara, T., and Honjo, T., (1995) *Genomics*, 28, 495-500 and Bleul, C.C., Fuhrbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A., (1996) *J. Exp. Med.* 184, 1101-1109). A basic physiological role for SDF-1 is implied by the high  
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level of conservation of the SDF-1 sequence between species. *In vitro*, SDF-1 stimulates chemotaxis of a wide range of cells including monocytes and bone marrow derived progenitor cells (Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Guierez-Ramos, J.C., (1996) *J. Exp. Med.* 185, 111-120 and Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A., (1996) *J. Exp. Med.* 184, 1101-1109). SDF-1 also stimulates a high percentage of resting and activated T-lymphocytes (Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A., (1996) *J. Exp. Med.* 184, 1101-1109 and Campbell, J.J., Hendrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A., and Butcher, E.C., (1998) *Science*, 279 381-383).

A variety of diseases require treatment with agents which are preferentially cytotoxic to dividing cells. Cancer cells, for example, may be targeted with cytotoxic doses of radiation or chemotherapeutic agents. A significant side-effect of this approach to cancer therapy is the pathological impact of such treatments on rapidly dividing normal cells. These normal cells may for example include hair follicles, mucosal cells and the hematopoietic cells, such as primitive bone marrow progenitor cells and stem cells. The indiscriminate destruction of hematopoietic stem, progenitor or precursor cells can lead to a reduction in normal mature blood cell counts, such as leukocytes, lymphocytes and red blood cells. A major impact on mature cell numbers may be seen particularly with neutrophils (neutropaenia) and platelets (thrombocytopenia), cells which naturally have relatively short half-lives. A decrease in leukocyte count, with concomitant loss of immune system function, may increase a patient's risk of opportunistic infection. Neutropaenia resulting from chemotherapy may for example occur within two or three days of cytotoxic treatments, and may leave the patient vulnerable to infection for up to 2 weeks until the hematopoietic system has recovered sufficiently to regenerate neutrophil counts. A reduced leukocyte count (leukopenia) as a result of cancer therapy may become sufficiently serious that therapy must be interrupted to allow the white blood cell count to rebuild. Interruption of cancer therapy can in turn lead to survival of cancer cells, an increase in the

incidence of drug resistance in cancer cells, and ultimately in cancer relapse. There is accordingly a need for therapeutic agents and treatments which facilitate the preservation of hematopoietic progenitor or stem cells in patients subject to treatment with cytotoxic agents.

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Bone marrow transplantation has been used in the treatment of a variety of hematological, autoimmune and malignant diseases. In conjunction with bone marrow transplantation, ex vivo hematopoietic (bone marrow) cell culture may be used to expand the population of hematopoietic progenitor or stem cells. It may be desirable to purge an ex vivo hematopoietic cell culture of cancer cells with cytotoxic treatments, while preserving the viability of the hematopoietic progenitor or stem cells. There is accordingly a need for agents and methods, which facilitate the preservation of hematopoietic progenitor or stem cells in ex vivo cell cultures exposed to cytotoxic agents.

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15 A number of proteins have been identified as inhibitors of hematopoietic progenitor cell development, with potential therapeutic usefulness as inhibitors of hematopoietic cell multiplication: macrophage inflammatory protein 1-alpha (MIP-1-alpha) and LD78 (see U.S. Patent No. 5,856,301); the alpha globin chain of hemoglobin and beta globin chain of hemoglobin (see U.S. Patent No. 6,022,848); and, interferon gamma (see U.S. Patent No. 5,807,744).

20 Permanent marrow recovery after cytotoxic drug and radiation therapy depends on the survival of hematopoietic stem cells having long term reconstituting (LTR) potential. The major dose limiting sequelae consequent to chemotherapy and/or radiation therapy are neutropenia and thrombocytopenia. Protocols involving dose intensification (i.e., to increase the log-kill of the respective tumour therapy) or schedule compression will exacerbate the degree and duration of myelosuppression associated with the standard chemotherapy and/or radiation therapy. For instance, in the adjuvant setting, repeated cycles of doxorubicin-based treatment have been

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shown to produce cumulative and long-lasting damage in the bone marrow progenitor cell populations (Lorhrman *et al.* (1978) Br. J. Haematol. 40:369). The effects of short-term hematopoietic cell damage resulting from chemotherapy has been overcome to some extent by the concurrent use of 5 G-CSF (Neupogen®), used to accelerate the regeneration of neutrophils (Le Chevalier (1994) Eur. J. Cancer 30A:410). This approach has been met with limitations also, as it is accompanied by progressive thrombocytopenia and cumulative bone marrow damage as reflected by a reduction in the quality of mobilized progenitor cells over successive cycles of treatment. Because of 10 the current interest in chemotherapy dose intensification as a means of improving tumour response rates and perhaps patient survival, the necessity for alternative therapies to either improve or replace current treatments to rescue the myeloablative effects of chemotherapy and/or radiation therapy has escalated, and is currently one of the major rate limiting factors for tumour 15 therapy dose escalations.

Transplanted peripheral blood stem cells (PBSC, or autologous PBSC) may provide a rapid and sustained hematopoietic recovery after the administration of high-dose chemotherapy or radiation therapy in patients with 20 hematological malignancies and solid tumours. PBSC transplantation has become the preferred source of stem cells for autologous transplantation because of the shorter time to engraftment and the lack of a need for surgical procedure necessary for bone marrow harvesting (Demirer *et al.* (1996) Stem Cells 14:106-116; Pettengel *et al.* (1992) Blood 82:2239-2248). Although the 25 mechanism of stem cell release into the peripheral blood from the bone marrow is not well understood, agents that augment the mobilization of CD34<sup>+</sup> cells may prove to be effective in enhancing autologous PBSC transplantation. G-CSF and GM-CSF are currently the most commonly used hematopoietic growth factors for PBSC mobilization, although the mobilized 30 cellular profiles can differ significantly from patient to patient. Therefore, other agents are required for this clinical application.

It is generally accepted that stem cell transplants for autoimmune disease should be initiated using autologous or allogenic grafts, where the former would be preferable since they may bear less risk of complication (Burt and Taylor (1999) *Stem Cells* 17:366-372). Lymphocyte depletion has also been recommended, where lymphocyte depletion is a form of purging autoreactive cells from the graft. In practice, aggressive lymphocyte depletion of an allograft can prevent alloreactivity (i.e., graft-versus-host disease (GVHD)) even without immunosuppressive prophylaxis. Therefore, a lymphocyte-depleted autograft may prevent recurrence of autoreactivity. As a consequence, any concurrent therapy that may enhance the survival of the CFU-GEMM myeloid stem cells, or BFU-E and CFU-GM myelomonocytic stem cells may be beneficial in therapies for autoimmune diseases where hematopoietic stem cells could be compromised.

Retrovirus-mediated gene transfer into murine hematopoietic stem cells and reconstitution of syngeneic mice have demonstrated persistence and functioning of the transgenes over extended period of time (Kume *et al.* (1999) 69:227-233). Terminally differentiated cells are relatively short-lived, except for memory B and T lymphocytes, and a large number of blood cells are replaced daily. Therefore, when long-term functional correction of blood cells by gene transfer is required, the target cells may be hematopoietic stem cells (Kume *et al.* (1999) 69:227-233). Compounds that can maintain the survival of the progenitor stem cells may therefore increase the efficiency of the gene transfer in that a greater population of hematopoietic stems cells are available.

A number of proteins have been identified and are currently being utilized clinically as inhibitors of hematopoietic progenitor cell development and hematopoietic cell proliferation (multiplication). These include GM-recombinant-methionyl human G-CSF (Neupogen<sup>®</sup>, Filgastim; Amgen), GM-CSF (Leukine<sup>®</sup>, Sargramostim; Immunex), erythropoietin (rhEPO, EpoGen<sup>®</sup>; Amgen), thrombopoietin (rhTPO; Genentech), interleukin-11 (rhIL-11, Amgen),

Neumega®; American Home Products), Flt3 ligand (Mobista; Immunex), multilineage hematopoietic factor (MARstem™; Maret Pharm.), myelopoietin (Leridistem; Searle), IL-3, myeloid progenitor inhibitory factor-1 (Mirostipen; Human Genome Sciences), stem cell factor (rhSCF, Stemgen®; Amgen).

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of Ara-C (350 mg/kg) on White Blood Cell Count (WBC) in mice in the presence (triangular data points, solid line, designated Ara-C + CTCE0021 in the legend) and absence (circular data points, dashed line, designated Ara-C in the legend) of a peptide of the invention (designated CTCE0021 and described in Examples 1 and 3).

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Figure 2A shows a concentration-dependant inhibition of  $^{125}\text{I}$ -SDF-1 binding to CXCR4 by SDF-1, obtained as described for the data shown in Figure 2A, indicating the affinity of SDF-1 for the CXCR4 receptor.

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Figure 2B shows the CXCR4 receptor binding of SDF-1 and the SDF-1 peptide agonist analogs. SDF-1 and the indicated analogs (competing ligands, described in Examples) were added at the concentrations illustrated in the presence of 4nM  $^{125}\text{I}$ -SDF-1. CEM cells were assessed for  $^{125}\text{I}$ -SDF-1 binding following 2 hr of incubation. The results are expressed as percentages of the maximal specific binding that was determined without competing ligand, and are the mean of three independent experiments.

Figure 3 shows the induction of  $[\text{Ca}^{2+}]_i$  mobilization by SDF-1 and SDF-1 receptor analogs (described in Examples). Fura-2,AM loaded THP-1 cells ( $1 \times 10^6/\text{ml}$ ) were stimulated with SDF-1, CTCE0021 or CTCE0022 at the concentrations indicated. The values represent the mean +/- one S.D. of n=3 experiments.

Figure 4 shows the induction of  $[\text{Ca}^{2+}]_i$  mobilization by SDF-1 and SDF-1 analogs. Fura-2,AM loaded THP-1 cells ( $1 \times 10^6/\text{ml}$ ) were stimulated with

native SDF-1 and the SDF-1 peptide analogs at the concentration of native SDF-1 concentration that gave the maximum  $[Ca^{2+}]_i$  stimulation ( $1\mu M$ ). The values represent the mean +/- one S.D. of  $n=3$  experiments. The designated compounds are as follows: SDF-1, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021), SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide (CTCE0022), SDF-1 (1-9)<sub>2</sub>-C9/C9-cysteine dimer (CTCE9901), SDF-1(1-17) (CTCE9902), SDF-1 (1-8)<sub>2</sub>-lysine bridge dimer (CTCE9904) and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) amide (CTCE0017).

Figure 5 shows cyclic proliferative activity of primitive normal colony forming cells (CFC) in the adherent layer of a standard long term culture (LTC), in which circles represent BFU-E cells (burst forming unit-erythroid precursors), and squares represent CFU-GM cells (colony forming unit – granulocyte-monocyte common precursor), illustrating the inhibitory effect of SDF-1 on cellular proliferation as measured by the susceptibility of the cells to an agent preferentially cytotoxic to proliferating cells.

Figure 6 shows cyclic proliferative activity of primitive normal CFC in the adherent layer of standard LTC, when treated with SDF-1, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)- K20/D24-cyclic amide (Compound #1), SDF-1(1-9)<sub>2</sub> (Compound #3), as measured by the susceptibility of the cells to an agent preferentially cytotoxic to dividing cells.

Figure 7 shows the effect of SDF-1 and SDF-1 analogs (defined in Examples) on the cycling of human progenitors from fetal liver transplanted NOD/SCID mice. The cycling status of mature and primitive colony forming cells (CFU-GM; colony forming unit-granulocyte-monocyte precursor, BFU-E; burst forming unit-erythroid precursor) in the suspension of  $CD34^+$  cells isolated from the marrow of transplanted NOD/SCID mice was determined by assessing the proportion of these progenitors that were inactivated (killed) by short term (20 min) or overnight (LTC-IC;long-term culture initiating cell) exposure of the cells to  $20\mu g/ml$  of high specific activity  $^3H$ -thymidine. Values

represent the mean +/- the S.D. of data from up to four experiments with up to four mice per point in each.

Figure 8 shows data indicating that SDF-1 enhances the detectability of CRU (colony regenerating units) regenerated in NOD/SCID Mice transplanted with human fetal liver.

Figure 9 shows the effect of SDF-1 and SDF-1 Agonists (defined in Examples) on the engraftment of human cells in human fetal liver transplanted NOD/SCID mice. A comparison of the number of phenotypically defined hematopoietic cells detected in the long bones (tibias and femurs) of mice four weeks after being transplanted with  $10^7$  light-density human fetal liver blood cells and then administered SDF-1, CTCE0021 or CTCE 0013 (0.5 mg/kg) three times per week for two weeks before sacrifice. Values represent the mean +/- one S.D. of results obtained from three to seven individual mice in three experiments.

Figure 10 shows the effect of CTCE0021 (1mg/kg, defined in the Examples) on the recovery of leukocytes following myeloablative chemotherapy with Ara-C (300mg/kg). Mice were treated with Ara-C alone (Ara-C) or in combination with CTCE0021. The results represent the mean +/- one S.D. of 6 animals/group.

Figure 11 shows the effect of CTCE0021 (defined in Examples) and Neupogen® (G-CSF) on the growth of white blood cells in Ara-C treated mice. C3Hhen mice (female) were treated with 500mg/kg Ara-C for two cycles - on days 0 and 10. During the second cycle of Ara-C dosing, Ara-C treated mice were injected with 10mg/kg CTCE0021, 10mg/kg Neupogen®, alone or together (on days -1, 0, and 1 to 3). Control represents animals treated with Ara-C alone. Blood was collected from the tail vein into heparin-containing tubes at the onset of the experiment, and one day before and 1, 7 and 12

days following the second Ara-C dose. A total white blood cell count was obtained. The results represent the mean +/- one S.D. of 6 animals/group.

Figure 12 shows the effect of CTCE0021 and Neupogen® on the relative growth of white blood cells in Ara-C treated mice. C3Hhen mice (female) were treated with 500mg/kg Ara-C for two cycles - on days 0 and 10. During the second cycle of Ara-C dosing, Ara-C treated mice were injected with 10mg/kg CTCE0021 (defined in Examples), 10mg/kg Neupogen®, alone or together (on days -1, 0, and 1 to 3). Control represents animals treated with Ara-C alone. Blood was collected from the tail vein into heparin-containing tubes at the onset of the experiment, and one day before 7 and 12 days following the second Ara-C dose. A total white blood cell count was obtained. The increase in leukocytes (white blood cells) was determined relative to the number of cells counted the day before the second cycle Ara-C dose was administered. The results represent the mean +/- one S.D. of 6 animals/group.

Figure 13 shows the sequences of human SDF-1alpha, SDF-1 Precursor (PBSF) and SDF-1beta.

## SUMMARY OF THE INVENTION

In accordance with various aspects of the invention, CXCR4 agonists may be used to treat bone marrow progenitor or stem cells to reduce the susceptibility of the cells to cytotoxic agents. CXCR4 agonists may be used to treat bone marrow progenitor cells or stem cells to reduce the rate of cellular multiplication. CXCR4 agonists may for example be used *in vivo* or *ex vivo* in bone marrow or peripheral blood stem cell transplantation procedures to treat bone marrow progenitor or stem cells. CXCR4 agonists may be used to treat cancer in a mammal in conjunction with one or more cytotoxic agents. Cytotoxic agents may for example include chemotherapeutic agents or radiation. CXCR4 agonists may be used therapeutically to regulate bone marrow progenitor or stem cell growth *in vivo*, *ex vivo* and in human diseases, such as cancer.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with some aspects of the invention, hematopoietic stem cells may be affected by CXCR4 agonists via a mechanism of cell growth repression. Since cytotoxic therapies utilized to kill proliferating cancerous cells, such as chemotherapeutic and/or radiation therapy, target proliferating cells, the CXCR4 agonists in accordance with various aspects of this invention may be utilized to reduce cytotoxin mediated destruction of hematopoietic cells, such as primitive bone marrow and peripheral blood progenitor and stem cells, and thereby to enhance recovery of mature blood cell counts, such as leukocytes, lymphocytes and red blood cells, following cytotoxin treatments. In various aspects of the invention, CXCR4 agonists may be given to the patient prior to, during or both prior to and during cytotoxin treatments, such as myeloablative chemotherapy and/or radiation therapy, in order to suppress the growth of the progenitor cells, and thus rescue them from destruction by the therapeutic regimen that the patient is being treated with, for example to treat a cancer. Therefore, cancers susceptible to treatment with CXCR4 agonists in accordance with various aspects of the invention may include both primary and metastatic tumors, and solid tumors, including carcinomas of breast, colon, rectum, lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gallbladder and bile ducts, small intestine, urinary tract (including kidney, bladder and urothelium), female genital tract, (including cervix, uterus, and ovaries as well as choriocarcinoma and gestational trophoblastic disease), male genital tract (including prostate, seminal vesicles, testes and germ cell tumors), endocrine glands (including the thyroid, adrenal, and pituitary glands), and skin, as well as hemangiomas, melanomas, sarcomas (including those arising from bone as well as Kaposi's sarcoma) and tumors of the brain, nerves, and soft tissues as well as astrocytomas, gliomas, glioblastomas, eyes, and meninges (including retinoblastomas, neuromas, neuroblastomas, Schwannomas, and meningiomas). In some aspects of the invention, CXCR4 agonists may also be useful in treating tumors, such as solid tumors, arising from hematopoietic

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5 malignancies such as leukemias (i.e. chloromas, plasmacytomas and the plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia) as well as in the treatment of lymphomas (both Hodgkin's and non-Hodgkin's lymphomas). In addition, CXCR4 agonists may  
10 be useful in the prevention of metastases from the tumors described above either when used alone or in combination with cytotoxic agents such as radiotherapy or chemotherapeutic agents.

10 In alternative aspects of the invention, CXCR4 agonists may be used to  
enrich populations of CD34<sup>+</sup> progenitor cells. Such cells may for example be  
enriched by CXCR4 agonists in bone marrow (BM) and peripheral blood (PB)  
15 stem cell transplantation procedures. Such procedures may be used to treat a  
variety of diseases (see for example Ball, E.D., Lister, J., and Law, P.  
Hematopoietic Stem Cell Therapy, Chruchill Livingston (of Harcourt Inc.), New  
York (2000)). CXCR4 agonists may accordingly be used in such  
15 hematopoietic stem cell transplantation (HSCT) protocols for the purpose of  
treating diseases, such as the following diseases that may be treated with  
CXCR4 agonists:

20 Aplastic Anemia;  
Acute Lymphoblastic Anemia.;  
Acute Myelogenous Leukemia;  
Myelodysplasia;  
Multiple Myeloma;  
Chronic Lymphocytic Leukemia;  
25 Congenital Immunodeficiencies (such as Autoimmune Lymphoproliferative  
disease, Wiscott-Aldrich Syndrome, X-linked Lymphoproliferative  
disease, Chronic Granulomatous disease, Kostmann Neutropenia,  
Leukocyte Adhesion Deficiency);

30 Metabolic Diseases (for instance those which have been HSCT indicated  
such as Hurler Syndrome (MPS I/II), Sly Syndrome (MPS VII),  
Childhood onset cerebral X-adrenoleukodystrophy, Globard-cell  
Leukodystrophy),

In alternative embodiments, CXCR4 agonists may be used to treat a variety of hematopoietic cells, and such cells may be isolated or may form only part of a treated cell population *in vivo* or *in vitro*. Cells amenable to treatment with CXCR4 agonists may for example include cells in the hematopoietic lineage, beginning with pluripotent stem cells, such as bone marrow stem or progenitor cells, lymphoid stem or progenitor cells, myeloid stem cells, CFU-GEMM cells (colony-forming-unit granulocyte, erythroid, 5 macrophage, megakaryocyte), B stem cells, T stem cells, DC stem cells, pre-B cells, prothymocytes, BFU-E cells (burst-forming unit - erythroid), BFU-MK cells, 10 CFU-GM cells (colony-forming cells (burst-forming unit - megakaryocytes), CFU-bas cells (colony-forming unit - unit - granulocyte-macrophage), CFU-Mast cells (colony forming unit - mast cell), CFU-G cells basophil), CFU-Mast cells (colony forming unit - mast cell), CFU-G cells 15 (colony forming unit granulocyte), CFU-M/DC cells (colony forming unit monocyte/dendritic cell), CFU-Eo cells (colony forming unit eosinophil), CFU-E cells (colony forming unit erythroid), CFU-MK cells (colony forming unit megakaryocyte), myeloblasts, monoblasts, B-lymphoblasts, T-lymphoblasts, 20 proerythroblasts, neutrophilic myelocytes, promonocytes, or other hematopoietic cells that differentiate to give rise to mature cells such as macrophages, myeloid related dendritic cells, mast cells, plasma cells, erythrocytes, platelets, neutrophils, monocytes, eosinophils, basophils, B-cells, T-cells or lymphoid related dendritic cells.

In some embodiments, the present invention is concerned with 25 polypeptides having the amino acid sequences shown in SEQ ID. NO.'s 1, 2 or 3 (Figure 13). In some embodiments, a pegylation moiety may be provided at any position on the sequence. The polypeptides of the present invention may include polypeptides in which part of the amino acid sequence is omitted, or polypeptides that consist of sequences containing additional or replaced 30 amino acids, or spliced forms of the sequences, yet induce activation of the CXCR4. In some embodiments, polypeptides of the invention may be at least 70%, 80% 90% or 95% identical to the polypeptides of Seq. ID. No.'s 1, 2 or

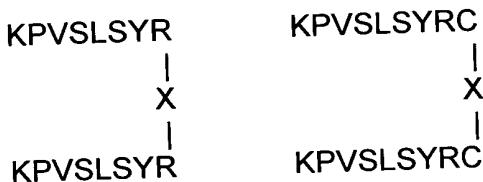
3, when optimally aligned, over a region of at least 10, 15, 20, 30, 40, 50, 60  
or 80 or more, contiguous amino acids. In alternative embodiments, SDF-1  
polypeptides of the invention may consist of amino acid sequences that are  
less than 70% identical to portions of SEQ ID No.'s 1, 2 or 3, where a  
5 polypeptide demonstrates CXCR4 agonist activity, such as activity that is  
comparable (such as within 0.01-, 0.1-, 1.0-, 10-, or 100-fold) to that obtained  
with the SDF-1 polypeptides of Seq. ID. No.'s 1, 2 or 3.

In one aspect of the invention, a putative SDF-1 polypeptide having  
10 some similarity to SEQ ID No.'s 1, 2 or 3 may be assessed for CXCR4 agonist  
activity. Putative SDF-1 polypeptides of the invention may for example be  
assayed for CXCR4 receptor binding as determined by receptor binding  
assays, such as radiolabeled ligand receptor competition assays. Activation  
15 of CXCR4 by an SDF-1 polypeptide may be determined through assaying  
activation of the receptor through standard biochemical techniques including  
intracellular calcium mobilization, cellular chemotaxis, chemiluminescence,  
degranulation assays, measurement of NADPH-dependent formation of  
reactive oxygen species, activation of secondary messenger enzymes such  
as G proteins, phospholipase C (PLC), protein kinase C (PKC), or of Src and  
20 Src family kinases (i.e., Fyn). In some embodiments, CXCR4 agonist activity,  
CXCR4 receptor binding or CXCR4 receptor activation of a putative CXCR4  
agonist of the invention may be at least 0.01-, 0.1-, 1.0-, 10-, or 100-fold of the  
corresponding parameter of a polypeptides of Seq. ID. No.'s 1, 2 or 3.

25 In alternative embodiments, a variety of small SDF-1 peptide  
analogues may be used as CXCR4 agonists. One such peptide is a dimer of  
amino acids 1-9, in which the amino acid chains are joined by a disulphide  
bond between each of the cysteines at position 9 in each sequence  
(designated SDF-1(1-9)<sub>2</sub> or KPVSLSYRC-CRYSLVPK). An alternative  
30 peptide is a dimer of amino acids 1-8, KPVSLSYR-X-RYSLVPK, in which  
the amino acid chains are joined by a linking moiety X between each of the  
arginines at position 8 in each sequence (designated SDF-1(1-8)<sub>2</sub>). CXCR4

agonist peptides may for example be selected from the group consisting of peptides having the following sequences:

5 KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCID  
PKLKWIQEYLEKALN; KPVSLSYRCPCRFFESH; KPVSLSYRC;  
10 KPVSLSYRC-CRYSLVPK; KPVSLSYRC-X-CRYSLVPK; and,  
KPVSLSYR-X-RYSLVPK. In the foregoing peptides X may be lysine with both the  $\alpha$  (alpha) and  $\epsilon$  (epsilon) amino groups of the lysine being associated with covalent (amide) bond formation and the lysyl carboxyl group being protected. The last two compounds in the forgoing list may, for example, be represented as follows, showing the peptide sequences in the standard 15 amino-to-carboxyl orientation:



20 In various alternative embodiments, such SDF-1 peptide analogs, along with SDF-1 polypeptides, are included amongst CXCR4 agonists of the invention.

25 In some embodiments, the CXCR4 agonists for use in the invention may be substantially purified peptide fragments, modified peptide fragments, analogues or pharmacologically acceptable salts of either SDF-1 $\alpha$  or SDF-1 $\beta$ .  
30 SDF-1 derived peptide agonists of CXCR4 may be identified by known biological assays and a variety of techniques such as the aforementioned or as discussed in Crump et al., 1997, The EMBO Journal 16(23) 6996-7007; and Heveker et al., 1998, Current Biology 8(7): 369-376; each of which are incorporated herein by reference. Such SDF-1 derived peptides may include homologs of native SDF-1, such as naturally occurring isoforms or genetic variants, or polypeptides having substantial sequence similarity to SDF-1, such as 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% sequence identity to at least a portion of the native SDF-1 sequence, the portion of native SDF-1 being any contiguous sequence of 10, 15, 20, 30, 40, 50 or more amino acids,

provided the peptides have CXCR4 agonist activity. In some embodiments, chemically similar amino acids may be substituted for amino acids in the native SDF-1 sequence (to provide conservative amino acid substitutions). In some embodiments, peptides having an N-terminal LSY sequence motif within 10, or 7, amino acids of the N-terminus, and/or an N-terminal RFFESH (SEQ ID NO:5) sequence motif within 20 amino acids of the N-terminus may be used provided they have CXCR4 agonistic activity. One family of such peptide agonist candidates has an LSY motif at amino acids 5-7. Alternative peptides further include the RFFESH (SEQ ID NO: 5) motif at amino acids 12-17. In alternative embodiments, the LSY motif is located at positions 3-5 of a peptide. The invention also provides peptide dimers having two amino acid sequences, which may each have the foregoing sequence elements, attached by a disulfide bridge within 20, or preferably within 10, amino acids of the N terminus, linking cysteine residues or  $\alpha$ -aminobutyric acid residues.

15 The invention further provides pharmaceutical compositions containing CXCR4 agonists. In one embodiment, such compositions include a CXCR4 agonist compound in a therapeutically or prophylactically effective amount sufficient to alter bone marrow and/or peripheral progenitor or stem cell growth, maturation and/or mobilization, and a pharmaceutically acceptable carrier. In another embodiment, the composition includes a CXCR4 agonist compound in a therapeutically or prophylactically effective amount sufficient to inhibit a cytotoxic effect of a cytotoxic agent, such as cytotoxic agents used in chemotherapy or radiation treatment of cancer, and a pharmaceutically acceptable carrier.

30 An "effective amount" of a compound of the invention includes a therapeutically effective amount or a prophylactically effective amount. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduction of bone marrow progenitor or stem cell multiplication, or reduction or inhibition of a cytotoxic effect of a cytotoxic agent. A

therapeutically effective amount of CXCR4 agonist may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the CXCR4 agonist to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the CXCR4 agonist are outweighed by the therapeutically beneficial effects.

A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting a cytotoxic effect of a cytotoxic agent. Typically, a prophylactic dose is used in subjects prior to or at an earlier stage of disease, so that a prophylactically effective amount may be less than a therapeutically effective amount.

In particular embodiments, a preferred range for therapeutically or prophylactically effective amounts of CXCR4 agonists may be 0.1 nM-0.1M, 0.1 nM-0.05M, 0.05 nM-15 $\mu$ M or 0.01 nM-10 M. It is to be noted that dosage values may vary with the severity of the condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and do not limit the dosage ranges that may be selected by medical practitioners.

The amount of active compound in the composition may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It may be advantageous to formulate parenteral compositions in

dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

As used herein "pharmaceutically acceptable carrier" or "excipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Alternatively, the carrier can be suitable for intravenous, intraperitoneal, intramuscular, sublingual or oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

In some embodiments, CXCR4 agonists may be formulated in pharmaceutical compositions with additional active ingredients, or administered in methods of treatment in conjunction with treatment with one or more additional medications, such as a medicament selected from the following: recombinant-methionyl human G-CSF (Neupogen®, Filgastim; Amgen), GM-CSF (Leukine®, Sargramostim; Immunex), erythropoietin

(rhEPO, EpoGen®; Amgen), thrombopoietin (rhTPO; Genentech), interleukin-11 (rhIL-11, Neumega®; American Home Products), Flt3 ligand (Mobista; Immunex), multilineage hematopoietic factor (MARstem™; Maret Pharm.), myelopoietin (Leridistem; Searle), IL-3, myeloid progenitor inhibitory factor-1 (Mirostipen; Human Genome Sciences), and stem cell factor (rhSCF, Stemgen®; Amgen).

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the CXCR4 agonists may be administered in a slow release formulation, for example in a composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. In accordance with an alternative aspect of the invention, a CXCR4 agonist may be formulated with one or more additional compounds that enhance the solubility of the CXCR4 agonist.

CXCR4 antagonist compounds of the invention may include SDF-1 derivatives, such as C-terminal hydroxymethyl derivatives, O-modified derivatives (e.g., C-terminal hydroxymethyl benzyl ether), N-terminally modified derivatives including substituted amides such as alkylamides and hydrazides and compounds in which a C-terminal phenylalanine residue is replaced with a phenethylamide analogue (e.g., Ser-Ile-phenethylamide as an analogue of the tripeptide Ser-Ile-Phe).

Within a CXCR4 agonist of the invention, a peptidic structure (such as an SDF-1 derived peptide) maybe coupled directly or indirectly to at least one modifying group. The term "modifying group" is intended to include structures that are directly attached to the peptidic structure (e.g., by covalent bonding or covalent coupling), as well as those that are indirectly attached to the peptidic structure (e.g., by a stable non-covalent bond association or by covalent coupling to additional amino acid residues, or mimetics, analogues or derivatives thereof, which may flank the SDF-1 core peptidic structure). For example, the modifying group can be coupled to the amino-terminus or carboxy-terminus of an SDF-1 peptidic structure, or to a peptidic or

peptidomimetic region flanking the core domain. Alternatively, the modifying group can be coupled to a side chain of at least one amino acid residue of a SDF-1 peptidic structure, or to a peptidic or peptido-mimetic region flanking the core domain (e.g., through the epsilon amino group of a lysyl residue(s), 5 through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain). Modifying groups covalently coupled to the peptidic structure can be attached by means and using methods well known in the art for linking 10 chemical structures, including, for example, amide, alkylamino, sulphide, carbamate or urea bonds.

In some embodiments, the modifying group may comprise a cyclic, heterocyclic or polycyclic group. The term "cyclic group", as used herein, 15 includes cyclic saturated or unsaturated (i.e., aromatic) group having from 3 to 10, 4 to 8, or 5 to 7 carbon atoms. Exemplary cyclic groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cyclooctyl. Cyclic groups may be unsubstituted or substituted at one or more ring positions. A cyclic group may for example be substituted with halogens, alkyls, cycloalkyls, 20 alkenyls, alkynyls, aryls, heterocycles, hydroxyls, aminos, nitros, thiols, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, sulfonates, selenoethers, ketones, aldehydes, esters,  $-CF_3$ ,  $-CN$ .

25 The term "heterocyclic group" includes cyclic saturated, unsaturated, and aromatic groups having from 3 to 10, 4 to 8, or 5 to 7 carbon atoms, wherein the ring structure includes about one or more heteroatoms. Heterocyclic groups include pyrrolidine, oxolane, thiolane, imidazole, oxazole, piperidine, piperazine, morpholine. The heterocyclic ring may be substituted at 30 one or more positions with such substituents as, for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, other heterocycles, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines,

carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, -CF<sub>3</sub>, -CN. Heterocycles may also be bridged or fused to other cyclic groups as described below.

5        The term "polycyclic group" as used herein is intended to refer to two or more saturated, unsaturated or aromatic cyclic rings in which two or more carbons are common to two adjoining rings, so that the rings are "fused rings".  
10      Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycyclic group may be substituted with such substituents as described above, as for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, -CF<sub>3</sub>, or -CN.

15      The term "alkyl" refers to the radical of saturated aliphatic groups, including straight chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In some embodiments, a straight chain or branched chain alkyl has 20 or fewer carbon atoms in its backbone (C<sub>1</sub>-C<sub>20</sub> for straight chain, C<sub>3</sub>-C<sub>20</sub> for branched chain), or 10 or fewer carbon atoms. In some embodiments, cycloalkyls may have from 4-10 carbon atoms in their ring structure, such as 5, 6 or 7 carbon rings. Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, having from one to ten carbon atoms in its backbone structure.  
20      Likewise, "lower alkenyl" and "lower alkynyl" have chain lengths of ten or less carbons.  
25

30      The term "alkyl" (or "lower alkyl") as used throughout the specification and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, halogen, hydroxyl, carbonyl (such

5 as carboxyl, ketones (including alkylcarbonyl and arylcarbonyl groups), and esters (including alkyloxycarbonyl and aryloxycarbonyl groups), thiocarbonyl, acyloxy, alkoxyl, phosphoryl, phosphonate, phosphinate, amino, acylamino, amido, amidine, imino, cyano, nitro, azido, sulfhydryl, alkylthio, sulfate, sulfonate, sulfamoyl, sulfonamido, heterocyclyl, aralkyl, or an aromatic or heteroaromatic moiety. The moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of aminos, azidos, iminos, amidos, phosphoryls (including phosphonates and phosphinates), sulfonyls (including sulfates, sulfonamidos, sulfamoyls and sulfonates), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters),  $-CF_3$ ,  $-CN$  and the like. 10 Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls,  $-CF_3$ ,  $-CN$ , and the like. 15

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

20 The term "aralkyl", as used herein, refers to an alkyl or alkylenyl group substituted with at least one aryl group. Exemplary aralkyls include benzyl (i.e., phenylmethyl), 2-naphthylethyl, 2-(2-pyridyl)propyl, 5-dibenzosuberyl, and the like.

25 The term "alkylcarbonyl", as used herein, refers to  $-C(O)-alkyl$ .  
 Similarly, the term "arylcarbonyl" refers to  $-C(O)-aryl$ . The term "alkyloxycarbonyl", as used herein, refers to the group  $-C(O)-O-alkyl$ , and the term "aryloxycarbonyl" refers to  $-C(O)-O-aryl$ . The term "acyloxy" refers to  $-O-C(O)-R_7$ , in which  $R_7$  is alkyl, alkenyl, alkynyl, aryl, aralkyl or heterocyclyl.  
 30

The term "amino", as used herein, refers to  $-N(R_\alpha)(R_\beta)$ , in which  $R_\alpha$  and  $R_\beta$  are each independently hydrogen, alkyl, alkyenyl, alkynyl, aralkyl, aryl, or in which  $R_\alpha$  and  $R_\beta$  together with the nitrogen atom to which they are attached, form a ring having 4-8 atoms. Thus, the term "amino", as used herein, includes unsubstituted, monosubstituted (e.g., monoalkylamino or monoaryl amino), and disubstituted (e.g., dialkylamino or alkylaryl amino) amino groups. The term "amido" refers to  $-C(O)-N(R_8)(R_9)$ , in which  $R_8$  and  $R_9$  are as defined above. The term "acylamino" refers to  $-N(R'_8)C(O)-R_7$ , in which  $R_7$  is as defined above and  $R'_8$  is alkyl.

As used herein, the term "nitro" means  $-NO_2$ ; the term "halogen" designates  $-F$ ,  $-Cl$ ,  $-Br$  or  $-I$ ; the term "sulphydryl" means  $-SH$ ; and the term "hydroxyl" means  $-OH$ .

The term "aryl" as used herein includes 5-, 6- and 7-membered aromatic groups that may include from zero to four heteroatoms in the ring, for example, phenyl, pyrrolyl, furyl, thiophenyl, imidazolyl, oxazole, thiazolyl, triazolyl, pyrazolyl, pyridyl, pyrazinyl, pyridazinyl and pyrimidinyl, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with such substituents as described above, as for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulphydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, a heterocyclol, an aromatic or heteroaromatic moiety,  $-CF_3$ ,  $-CN$ , or the like. Aryl groups can also be part of a polycyclic group. For example, aryl groups include fused aromatic moieties such as naphthyl, anthracenyl, quinolyl, indolyl, and the like.

Modifying groups may include groups comprising biotinyl structures, fluorescein-containing groups, a diethylene-triaminepentaacetyl group, a (O)-menthoxyacetyl group, a N-acetylneuraminy group, a cholyl structure or an

iminiobiotinyl group. A CXCR4 agonist compound may be modified at its carboxy terminus with a cholyl group according to methods known in the art (see e.g., Wess, G. et al. (1993) *Tetrahedron Letters*, 34:817-822; Wess, G. et al. (1992) *Tetrahedron Letters* 33:195-198; and Kramer, W. et al. (1992) *J. Biol. Chem.* 267:18598-18604). Cholyl derivatives and analogues may also be used as modifying groups. For example, a preferred cholyl derivative is Aic (3-(O-aminoethyl-iso)-cholyl), which has a free amino group that can be used to further modify the CXCR4 agonist compound. A modifying group may be a "biotinyl structure", which includes biotinyl groups and analogues and derivatives thereof (such as a 2-iminobiotinyl group). In another embodiment, the modifying group may comprise a "fluorescein-containing group", such as a group derived from reacting an SDF-1 derived peptidic structure with 5-(and 6)-carboxyfluorescein, succinimidyl ester or fluorescein isothiocyanate. In various other embodiments, the modifying group(s) may comprise an N-acetylneuraminy group, a trans-4-cotininecarboxyl group, a 2-imino-1-imidazolidineacetyl group, an (S)-(-)-indoline-2-carboxyl group, a (-)-menthoxyacetyl group, a 2-norbornaneacetyl group, a  $\gamma$ -oxo-5-acenaphthenebutyryl, a (-)-2-oxo-4-thiazolidinecarboxyl group, a tetrahydro-3-furoyl group, a 2-iminobiotinyl group, a diethylenetriaminepentaacetyl group, a 4-morpholinecarbonyl group, a 2-thiopheneacetyl group or a 2-thiophenesulfonyl group.

A CXCR4 agonist compound of the invention may be further modified to alter the specific properties of the compound while retaining the desired functionality of the compound. For example, in one embodiment, the compound may be modified to alter a pharmacokinetic property of the compound, such as *in vivo* stability, bioavailability or half-life. The compound may be modified to label the compound with a detectable substance. The compound may be modified to couple the compound to an additional therapeutic moiety. To further chemically modify the compound, such as to alter its pharmacokinetic properties, reactive groups can be derivatized. For example, when the modifying group is attached to the amino-terminal end of

the SDF-1 core domain, the carboxy-terminal end of the compound may be further modified. Potential C-terminal modifications include those that reduce the ability of the compound to act as a substrate for carboxypeptidases. Examples of C-terminal modifiers include an amide group, an ethylamide group and various non-natural amino acids, such as D-amino acids and  $\beta$ -alanine. Alternatively, when the modifying group is attached to the carboxy-terminal end of the aggregation core domain, the amino-terminal end of the compound may be further modified, for example, to reduce the ability of the compound to act as a substrate for aminopeptidases.

- 10 A CXCR4 agonist compound can be further modified to label the compound by reacting the compound with a detectable substance. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials.
- 15 Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferon, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, 20 dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include  $^{14}\text{C}$ ,  $^{123}\text{I}$ ,  $^{124}\text{I}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ . A CXCR4 agonist compound may be radioactively labeled with  $^{14}\text{C}$ , either by incorporation of  $^{14}\text{C}$  into the modifying group or one or more amino acid structures in the CXCR4 agonist compound.
- 25 Labeled CXCR4 agonist compounds may be used to assess the *in vivo* pharmacokinetics of the compounds, as well as to detect disease progression or propensity of a subject to develop a disease, for example for diagnostic purposes. Tissue distribution CXCR4 receptors can be detected using a labeled CXCR4 agonist compound either *in vivo* or in an *in vitro* sample derived from a subject. For use as an *in vivo* diagnostic agent, a CXCR4 30 antagonist compound of the invention may be labeled with radioactive technetium or iodine. A modifying group can be chosen that provides a site at

which a chelation group for the label can be introduced, such as the Aic derivative of cholic acid, which has a free amino group. For example, a phenylalanine residue within the SDF-1 sequence (such as aminoacid residue 13) may be substituted with radioactive iodotyrosyl. Any of the various isotopes of radioactive iodine may be incorporated to create a diagnostic agent. <sup>123</sup>I (half-life=13.2 hours) may be used for whole body scintigraphy, <sup>124</sup>I (half life=4 days) may be used for positron emission tomography (PET), <sup>125</sup>I (half life=60 days) may be used for metabolic turnover studies and <sup>131</sup>I (half life=8 days) may be used for whole body counting and delayed low resolution imaging studies.

In an alternative chemical modification, a CXCR4 agonist compound of the invention may be prepared in a "prodrug" form, wherein the compound itself does not act as a CXCR4 agonist, but rather is capable of being transformed, upon metabolism *in vivo*, into a CXCR4 agonist compound as defined herein. For example, in this type of compound, the modifying group can be present in a prodrug form that is capable of being converted upon metabolism into the form of an active CXCR4 agonist. Such a prodrug form of a modifying group is referred to herein as a "secondary modifying group." A variety of strategies are known in the art for preparing peptide prodrugs that limit metabolism in order to optimise delivery of the active form of the peptide-based drug (see e.g., Moss, J. (1995) in Peptide-Based Drug Design: Controlling Transport and Metabolism, Taylor, M. D. and Amidon, G. L. (eds), Chapter 18.

CXCR4 agonist compounds of the invention may be prepared by standard techniques known in the art. A peptide or polypeptide component of a CXCR4 agonist may be composed, at least in part, of a peptide synthesized using standard techniques (such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993); Grant, G. A. (ed.). Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992); or Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B., and

Baggiolini, M., (1994) J. Biol. Chem., 269, 16075-16081). Automated peptide synthesizers are commercially available (e.g., Advanced ChemTech Model 396; Milligen/Bioscience 9600). Peptides and polypeptides may be assayed for CXCR4 agonist activity in accordance with standard methods. Peptides and polypeptides may be purified by HPLC and analyzed by mass spectrometry. Peptides and polypeptides may be dimerized via a disulfide bridge formed by gentle oxidation of the cysteines using 10% DMSO in water. Following HPLC purification dimer formation may be verified, by mass spectrometry. One or more modifying groups may be attached to a SDF-1 derived peptidic component by standard methods, for example using methods for reaction through an amino group (e.g., the alpha-amino group at the amino-terminus of a peptide), a carboxyl group (e.g., at the carboxy terminus of a peptide), a hydroxyl group (e.g., on a tyrosine, serine or threonine residue) or other suitable reactive group on an amino acid side chain (see e.g., Greene, T. W. and Wuts, P. G. M. *Protective Groups in Organic Synthesis*, John Wiley and Sons, Inc., New York (1991)).

In another aspect of the invention, CXCR4 agonist peptides may be prepared according to standard recombinant DNA techniques using a nucleic acid molecule encoding the peptide. A nucleotide sequence encoding the peptide or polypeptide may be determined using the genetic code and an oligonucleotide molecule having this nucleotide sequence may be synthesized by standard DNA synthesis methods (e.g., using an automated DNA synthesizer). Alternatively, a DNA molecule encoding a peptide compound may be derived from the natural precursor protein gene or cDNA (e.g., using the polymerase chain reaction (PCR) and/or restriction enzyme digestion) according to standard molecular biology techniques.

The invention also provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a peptide of the invention. In some embodiments, the peptide may comprise an amino acid sequence having at least one amino acid deletion compared to native SDF-1. The term

"nucleic acid molecule" is intended to include DNA molecules and RNA molecules and may be single-stranded or double-stranded. In alternative embodiments, the isolated nucleic acid encodes a peptide wherein one or more amino acids are deleted from the N-terminus, C-terminus and/or an internal site of SDF-1.

To facilitate expression of a peptide compound in a host cell by standard recombinant DNA techniques, the isolated nucleic acid encoding the peptide may be incorporated into a recombinant expression vector.

10 Accordingly, the invention also provides recombinant expression vectors comprising the nucleic acid molecules of the invention. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been operatively linked. Vectors may include circular double stranded DNA plasmids and/or viral vectors. Certain vectors are capable of autologous replication in a host cell into which they are introduced (such as bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (such as non-episomal mammalian vectors) may be integrated into the genome of a host cell upon introduction into the host cell, and thereby may be replicated along with the host genome. Certain vectors may be capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or "expression vectors".

In recombinant expression vectors of the invention, the nucleotide sequence encoding a peptide may be operatively linked to one or more regulatory sequences, selected on the basis of the host cells to be used for expression. The terms "operatively linked" or "operably" linked mean that the sequences encoding the peptide are linked to the regulatory sequence(s) in a manner that allows for expression of the peptide compound. The term "regulatory sequence" includes promoters, enhancers, polyadenylation signals and other expression control elements. Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods

in Enzymology 185, Academic Press, San Diego, Calif. (1990) (incorporated herein by reference). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell, those that direct expression of the nucleotide sequence only in certain host cells (such as tissue-specific regulatory sequences) and those that direct expression in a regulatable manner (such as only in the presence of an inducing agent). The design of the expression vector may depend on such factors as the choice of the host cell to be transformed and the level of expression of peptide compound desired.

10

The recombinant expression vectors of the invention may be designed for expression of peptide compounds in prokaryotic or eukaryotic cells. For example, peptide compounds may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector may be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase. Examples of vectors for expression in yeast *S. cerevisiae* include pYEPSec1 (Baldari et al., (1987) EMBO J. 6:229-234), pMFA (Kurjan and Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al., (1987) Gene 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of proteins or peptides in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al., (1983) Mol. Cell. Biol. 3:2156-2165) and the pVL series (Lucklow, V. A., and Summers, M. D., (1989) Virology 170:31-39). Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) Nature 329:840) and pMT2PC (Kaufman et al. (1987), EMBO J. 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

102740-2 07/08/2020

In addition to regulatory control sequences, recombinant expression vectors may contain additional nucleotide sequences, such as a selectable marker gene to identify host cells that have incorporated the vector. Selectable marker genes are well known in the art. To facilitate secretion of the peptide compound from a host cell, in particular mammalian host cells, the recombinant expression vector preferably encodes a signal sequence operatively linked to sequences encoding the amino-terminus of the peptide compound, such that upon expression, the peptide compound is synthesized with the signal sequence fused to its amino terminus. This signal sequence directs the peptide compound into the secretory pathway of the cell and is then cleaved, allowing for release of the mature peptide compound (i.e., the peptide compound without the signal sequence) from the host cell. Use of a signal sequence to facilitate secretion of proteins or peptides from mammalian host cells is well known in the art.

A recombinant expression vector comprising a nucleic acid encoding a peptide compound may be introduced into a host cell to produce the peptide compound in the host cell. Accordingly, the invention also provides host cells containing the recombinant expression vectors of the invention. The terms "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. A host cell may be any prokaryotic or eukaryotic cell. For example, a peptide compound may be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells. The peptide compound may be expressed *in vivo* in a subject to the subject by gene therapy (discussed further below).

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation, transfection or infection techniques. The terms

5 "transformation", "transfection" or "infection" refer to techniques for introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection and viral-mediated infection. Suitable methods for transforming, transfecting or infecting host cells can for example be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

10 Methods for introducing DNA into mammalian cells *in vivo* are also known, and may be used to deliver the vector DNA of the invention to a subject for gene therapy.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to 15 identify and select these integrants, a gene that encodes a selectable marker (such as resistance to antibiotics) may be introduced into the host cells along with the gene of interest. Preferred selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker may be introduced into a host cell 20 on the same vector as that encoding the peptide compound or may be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid may be identified by drug selection (cells that have incorporated the selectable marker gene will survive, while the other cells die).

25 A nucleic acid of the invention may be delivered to cells *in vivo* using methods such as direct injection of DNA, receptor-mediated DNA uptake or viral-mediated infection. Direct injection has been used to introduce naked DNA into cells *in vivo* (see e.g., Acsadi et al. (1991) Nature 332:815-818; Wolff et al. (1990) Science 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* may be used. Such an apparatus may be commercially available (e.g., from BioRad). Naked DNA 30 may also be introduced into cells by complexing the DNA to a cation, such as

polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C. H. (1988) J. Biol. Chem. 263:14621; Wilson et al. (1992) J. Biol. Chem. 267:963-967; and U.S. Pat. No. 5,166,320). Binding of the DNA-ligand complex to the receptor may facilitate uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids that disrupt endosomes, thereby releasing material into the cytoplasm, may be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel et al. (1991) Proc. Natl. Acad. Sci. USA 88:8850; Cristiano et al. (1993) Proc. Natl. Acad. Sci. USA 90:2122-2126).

Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for reviews see Miller, A. D. (1990) Blood 76:271, Kume et al. (1999) International. J. Hematol. 69:227-233). Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include .pψi.Crip, .pψi.Cre, .pψi.2 and .pψi.Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Pat. No. 4,868,116; U.S. Pat. No. 4,980,286; PCT Application WO

20250-20758360

89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). In various embodiments, a genome of a retrovirus that encodes and expresses a polypeptide compound of the invention, may be utilized for the propagation and/or survival of cells, such as 5 hematopoietic progenitor stem cells, for the purposes of maintaining and/or growing cells for the clinical purposes of blood transfusion or engraftment, host conditioning or applications relevant to chemotherapy, radiation therapy or myeloablative therapy.

10 For use as a gene therapy vector, the genome of an adenovirus may be manipulated so that it encodes and expresses a peptide compound of the invention, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 15 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are 20 advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited supra), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 25 89:2581-2584). In various embodiments, a genome of an adenovirus that encodes and expresses a polypeptide compound of the invention, may be utilized for the propagation and/or survival of cells, such as hematopoietic progenitor stem cells, stromal cells, or mesenchymal cells, for the purposes of maintaining and/or growing cells for the clinical purposes of blood transfusion or engraftment, host conditioning or applications relevant to chemotherapy, 30 radiation therapy or myeloablative therapy.

In some embodiments, adeno-associated virus (AAV) may be used as a gene therapy vector for delivery of DNA for gene therapy purposes. AAV is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle (Muzyczka et al. *Curr. Topics in Micro. and Immunol.* 5 (1992) 158:97-129). AAV may be used to integrate DNA into non-dividing cells (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). An AAV vector such as that described in 10 Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 may be used to introduce DNA into cells (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; USA 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790). In 15 some embodiments, a genome of an AAV that encodes and expresses a polypeptide compound of the invention, may be utilized for the propagation and/or survival of cells, such as hematopoietic progenitor stem cells, stromal cells or mesenchymal cells, for the purposes of maintaining and/or growing cells for the clinical purposes of blood transfusion or engraftment, host 20 cells for the clinical purposes of blood transfusion or engraftment, host conditioning or applications relevant to chemotherapy, radiation therapy or myeloablative therapy.

General methods for gene therapy are known in the art. See for example, U.S. Pat. No. 5,399,346 by Anderson et al. A biocompatible capsule 25 for delivering genetic material is described in PCT Publication WO 95/05452 by Baetge et al. Methods for grafting genetically modified cells to treat central nervous system disorders are described in U.S. Pat. No. 5,082,670 and in PCT Publications WO 90/06757 and WO 93/10234, all by Gage et al. Methods of gene transfer into hematopoietic cells have also previously been 30 reported (see Clapp, D. W., et al., *Blood* 78: 1132-1139 (1991); Anderson, *Science* 288:627-9 (2000); and , Cavazzana-Calvo et al., *Science* 288:669-72 (2000), all of which are incorporated herein by reference).

Cancers susceptible to treatment with CXCR4 agonists in accordance with various aspects of the invention may include both primary and metastatic tumors, such as solid tumors, including carcinomas of the breast, colon, rectum, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gall bladder and bile ducts, small intestine, urinary tract (including kidney, bladder, and urothelium), female genital tract (including cervix, uterus, and ovaries as well as choriocarcinoma and gestational trophoblast disease), male genital tract (including prostate, seminal vesicles, testes, and germ cell tumors), 10 endocrine glands (including the thyroid, adrenal and pituitary glands), and skin, as well as hemangiomas, melanomas, sarcomas (including those arising from bone and soft tissues as well as Kaposi's sarcoma) and tumors of the brain, nerves, eyes, and meninges (including astrocytomas, gliomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas, and 15 meningiomas). In some aspects of the invention, CXCR4 agonists may also serve in treating solid tumors arising from hematopoietic malignancies such as leukemias (i.e., chloromas, plasmacytomas and the plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia) as well as in the treatment of lymphoma (both Hodgkin's and non-Hodgkin's lymphomas). 20 In addition, CXCR4 agonists may be therapeutic in the prevention of metastasis from the tumors described above either when used alone or in combination with cytotoxic agents such as radiotherapy or chemotherapeutic agents.

25 In alternative aspects of the invention, CXCR4 agonists such as SDF-1 polypeptides may target CD34<sup>+</sup> cells to mediate release of CD34<sup>+</sup> cells to the peripheral blood. In these aspects of the invention, CXCR4 agonists such as SDF-1 may enhance circulating CD34<sup>+</sup> cell proliferation and hematopoietic stem or progenitor cell survival or levels, which may for example be useful in 30 stem cell transplantation.

In various aspects of the invention, CXCR4 agonists may be used in reducing the rate of hematopoietic cell multiplication. Method of the invention may comprise administration of an effective amount of CXCR4 agonists to cells selected from the group consisting of hematopoietic stem cells and hematopoietic progenitor cells, stromal cells or mesenchymal cells. In alternative embodiments, a therapeutically effective amount of the CXCR4 agonist may be administered to a patient in need of such treatment. Patients in need of such treatments may include, for example: patients having cancer, patients having an autoimmune disease, patients requiring functional gene transfer into hematopoietic stems cells, stromal cells or mesenchymal cells (such as for the dysfunction of any tissue or organ into which a stem cell may differentiate), patients requiring lymphocyte depletion, patients requiring depletion of a blood cancer in the form of purging autoreactive or cancerous cells using autologous or allogenic grafts, or patients requiring autologous peripheral blood stem cell transplantation. A patient in need of treatment in accordance with the invention may also be receiving cytotoxic treatments such as chemotherapy or radiation therapy. In some embodiments, CXCR4 agonists may be used in treatment to purge an ex vivo hematopoietic stem cell culture of cancer cells with cytotoxic treatment, while preserving the viability of the hematopoietic progenitor or stem cells.

In alternative embodiments, CXCR4 agonists may be used in accordance with the invention to treat hematopoietic cells, in patients in need of such treatment, for example:

- v) In immunotherapy of cancer and infectious disease;  
vi) In solid organ regeneration (Silberstein and Toy, 2001, JAMA Vol 285, 577-580);  
vii) In stem cell gene therapy and retro-virus gene transfer into  
5 hematopoietic progenitor cells (Hacein-Bey, 2001, Hum. Gene Ther. Vol 12, 291-301; Kaji and Leiden, 2001, JAMA Vol 285, 545-550), stromal cells, or mesenchymal cells;  
viii) In bone development, bone repair, and skeletal regeneration therapy.

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Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to".

20 **EXAMPLES**

The following examples illustrate, but do not limit, the present invention.

**Example 1**

25 Peptides of the invention may be synthesized chemically using the Fmoc/tBu strategy on a continuos flow peptide synthesizer, as for example has been carried out using the following protocols:

A) Reagents (solvents, support, chemicals)

30 Main Solvent: N,N-Dimethylformamide (DMF): certified ACS spectroanalyzed from Fisher (D131-4) M.W = 73.10. The DMF is treated with

activated molecular sieves, type 4A (from BDH: B54005) for at least two weeks then tested with FDNB (2,4-Dinitrofluorobenzene from Eastman).

5 Procedure: Mix equal volumes of FDNB solution (1mg/ml in 95% EtOH) and DMF; Let stand 30 minutes; read the absorbance at 381 nm over a FDNB blank (0.5ml FDNB + 0.5ml 95% EtOH). If the absorbance ~ 0.2, the DMF is suitable to be used for the synthesis.

Debloking Agent: 20% Piperidine (from Aldrich Chemical company, catalog No: 10,409-4) in DMF containing 0.5 % triton X100 v/v ( from Sigma , catalg No: T-9284 ).

10 Activating Agents: 2-(H-benzotriazol-yl) 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU: M.W.=321.09. from Quantum Richilieu, catalog No: R0139)/ Hydroxybenzotriazole (HOBr M.W.=135.1 from Quantum Richilieu, catalog No.: R0166-100) respectively, 0.52 M in DMF and 4-Methylmorpholine (NMM ; M.W.=101.15, d=0.926 from Aldrich, catalog No.: M5,655-7): 0.9 M in DMF or in the case of sensitive amino acids to racemization like Cys, we use 15 2,4,6-Collidine, 99% ( M.W.=121.18,d=0.917, from Aldrich, catalog No: 14,238-7 ): 0.78M in DMF/DCM, 1/1 v/v.

20 Support: TentaGel R RAM (90  $\mu$ m), RinK-type Fmoc (from Peptides International, catalog No.: RTS -9995-PI): 0.21 mmol/g, 0.5g for 0.1 mmol of peptide.

25 Fmoc-L-amino derivative, side-chains protected with: Boc; tBu; Trt groups: with 4 fold excess (from Peptides International, Bachem, Novabiochem, Chem-Impex Inc). Glu24 and Lys24 are Allyl-protected (from Millipore/Perseptive Biosystems).

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#### B) Initial Amino Loading and Peptide Synthesis Procedure

The first amino acid Asn31 and the remaining residues are doble coupled at 450°C automatically with 4-fold excess in each coupling. The synthesis is interrupted after residue Leu19. The peptide-bound support is removed from the synthesizer column and placed in a react-vial containing a small magnetic bar for gentle stirring.

### C) Removal of The Allyl Groups

C) Removal of the Allyl group:  
A solution of tetrakis(triphenylphosphine)Palladium(0) Pd(PPh<sub>3</sub>)<sub>4</sub> (from Sigma-Aldrich, catalog No: 21,666-6); M.W.=1155.58 x 0.1 mmol peptide x 3 fold = 347mg dissolved in 5% Acetic Acid; 2.5% NMM in CHCl<sub>3</sub> to 0.14 M, under argon. The solution is added to the support-bound peptide previously removed from the column in a reactvial containing a small mangnetic bar for gentle stirring. The mixture is flushed with argon, sealed and stirred at room temperature for 6 hours. The support-bound peptide is trasferred to a filter funnel, washed with 30 ml of a solution made of 0.5% Sodium Diethyldithiocarbonate/ in DMF the DCM; DCM/DMF (1 : 1) and DMF. A positive Kaiser test indicate the deprotection of the amino side chaine of the Lys20.

#### D) Lactam Formation:

15 Activating agent: 7-Azabenztriazol-1-yloxytris (pyrrolindino) phosphonium-hexafluorophosphate (PyAOP: M.W.=521.7 from PerSeptive Biosystems GmbH, catalog No: GEN076531) , 1.4-fold:  $0.105\text{mmol} \times 1.4 \times 521.7 = 76.6\text{mg}$  and NMM 1.5-fold:  $0.105 \times 1.4 \times 1.5 = 0.23 \text{ mmol} v = 0.23/0.9 \text{ M NMM solution} = 263 \mu\text{l}$ )

20 The cyclisation may be carried out in an amino acid vial at room  
temperature overnight (~16 hours) with gentle agitation. The completion of  
cyclization may be indicated by a negative kaiser test. The support-bound  
peptide may be poured into the column, washed with DMF and the  
synthesis continues to completion, with a cyclic amide bridge thereby  
25 introduced into the peptide.

#### E) Final Product Removal From The Support:

E) Final Product Removal: The support-bound peptide is removed from the synthesizer into a medium filter funnel, washed with DCM to replace the non-volatile DMF and thoroughly dried under high vacuum for at least two hours, or preferably, overnight.

#### Cleavage Mixture (reagent K):

TFA/Phenol/Water/Thio-Anisol/EDT (82/5/5/5/2.5) ; 7.5ml

Support: 0.5g resin-peptide.

TFA 6.15ml ( Biograde from Halocarbon )

Phenol 0.375ml ( Aldrich )

5 Water 0.375ml ( MillQ )

Thio-Anisol 0.375ml ( Aldrich )

EDT 0.187ml ( Aldrich )

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10 Total 7.5ml

15 The cleavage may be performed at room temperature for 4 hours  
with gentle agitation on a rocker.

20 F) Precipitation of The Peptide

25 The cleaved peptide solution is filtered through a filter funnel in a 50 ml round bottom flask. The support is rinsed twice with 4 ml TFA. The TFA solution is concentrated on a rotavap and added drop wise into a cold diethyl ether previously treated with activated neutral aluminum oxide to make it free of peroxide. Approximately 10-fold excess of ether are used.

30 The beads are stored until the yield is determined a peptide characterized. The precipitate is collected at room temperature in screw capped 50 ml polypropylene vial by centrifugation at 2K rpm, using a top bench centrifuge (4 minutes run time). The pellet is washed 3x with cold ether, centrifuged and dried with a flow of argon. The precipitate is dissolved in 20 % acetonitrile 0.1% TFA and lyophilized.

35 G) Crude Product Characterization:

The product is characterized by analytical HPLC.

Experimental conditions: Column: Vydac 218TP54: C18 reversed-phase

5µm, 4.6 mm ID x 150 mm L.

Eluants: 0.1% TFA/H<sub>2</sub>O (solvent A); 0.1% TFA/acetonitrile (solvent B)

Elution Conditions: 20-50% B (40 min); 60-90% B (5 min); 90-20% B (5 min); 20% B (10 min). At 1.0 ml/min and A214 nm = 0.5 absorbance unit full scale.

5 H) Sample Preparation:

An aliquot of the product is weighed and dissolved in 20% acetonitrile 10 0.1% TFA at a concentration of 2 mg/ml. The solution is microfuged and 20 $\mu$ l is applied onto the column. The main peak or the major peaks are collected, SpeedVac dried and molecular weight determined by mass spectrometry.

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In accordance with various aspects of the invention, a wide variety of peptide sequences may be prepared, for which the following nomenclature may be used. The portions of the peptide corresponding to a chemokine sequence, such as an SDF-1 sequence may be identified by specifying the corresponding portion of the chemokine, wherein for example a reference to an SDF-1 sequence refers to a sequence having substantial identity to a portion of the sequence of SEQ ID No: 1. For example, the nomenclature SDF-1(1-14) connotes the first fourteen amino acids of the N-terminal sequence of SDF-1 of SEQ ID No: 1. In some embodiments, N-terminal and C-terminal portions of an SDF-1 sequence may be linked by various amino acids, or other linking moieties, denoted by a formula (L) $n$ , wherein "L" is a linking moiety which may for example be an amino acid and  $n$  is zero or an integer. The carboxy terminal of the peptide may be modified to be an amide rather than a carboxylic acid. In some embodiments, polypeptides of the invention may be of the following formula:

SDF-1(1-X)-(L) $n$ -SDF-1(Y-Z)

wherein:

30

X is an integer from 5 to 20;

L is a linking moiety having at least one carbon atom, such as a substituted or unsubstituted alkyl moiety, or an amino acid;

$n$  is an integer from 1 to 50

Y is an integer from 50 to 60

Z is an integer from 60 to 67

5 In some embodiments,  $(CH_2)_n$  may for example be used as a linker (L) between N- and C-terminal, where n is an integer and may for example be less than 20, 30, 40, 50 or 100.

Exemplary embodiments of linear polypeptide sequences are as follows:

### SDF-1(1-14)-(G)<sub>3</sub>-SDF-1(55-67) acid:

H<sub>2</sub>NKPVSLSYRCPCRFFGGGLKWIQEYLEKALNCOOH

### SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) acid:

H<sub>2</sub>NKPVSLSYRCPCRFFGGGGGLKWIQEYLEKALNCOOH

### SDF-1(1-14)-(G)<sub>3</sub>-SDF-1(55-67) amide:

H<sub>2</sub>NKPVSLSYRCPCRFFGGGLKWIQEYLEKALNCONH<sub>2</sub>

### SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) amide:

H<sub>2</sub>NKPVSLSYRCPCRFFGGGGLKWIQEYLEKALNCONH<sub>2</sub>

SDF-1(1-17)-(G)<sub>3</sub>-SDF-1(55-67) acid:

H<sub>2</sub>NKPVSLSYRPCRFFESHGGGLKWIQEYLEKALNCOOH

SDF-1(1-17)-(G)<sub>4</sub>-SDF-1(55-67) acid:

H<sub>2</sub>NKPVSLSYRPCPCRFFESHGGGGLKWIQEYLEKALNCOOH

### SDF-1(1-17)-(G)<sub>3</sub>-SDF-1(55-67) amide:

H<sub>2</sub>NKPVSLSYRPCRFFESHGGGLKWIQEYLEKALNCONH<sub>2</sub>

### SDF-1(1-17)-(G)<sub>3</sub>-SDF-1(55-67) amide:

H<sub>2</sub>NKPVSLSYRCPCRFFESHGGGLKWIQEYLEKALNCONH<sub>2</sub>

In alternative embodiments, peptides of the invention may be cyclized, for example glutamate (E) and lysine (K) residues may be joined by side chain cyclization using a lactam formation procedure, as indicated in the following sequences by double underlining of linked residues. Lactams may for example be formed between glutamic acid (E) at amino acid residue 24 and lysine (K) at either position 20 or 28 in the polypeptide (which does not correspond necessarily with the numbering of that residue in the native sequence). In further alternatives, a lysine (K) may be substituted by leucine (L), ornithine (O) or other hydrophobic residues, such as isoleucine (I), norleucine (Nle), methionine (M), valine (V), alanine (A), tryptophan (W) or Phenylalanine (F). Similarly, glutamate (E) may for example be substituted with aspartate (D), denoted by nomenclature such as (E24 -> D) indicating a substitution at position 24 in the peptide wherein aspartate replaces glutamate.

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic acid

H<sub>2</sub>NKPVSLSYRCPCRFFGGGLKWIQEYLEKALNCOOH

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic acid

H<sub>2</sub>NKPVSLSYRCPCRFFGGGLKWIQEYLEKALNCOOH

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide

H<sub>2</sub>NKPVSLSYRCPCRFFGGGLKWIQEYLEKALNCONH<sub>2</sub>

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) K20/E24-cyclic amide

H<sub>2</sub>NKPVSLSYRCPCRFFGGGLKWIQEYLEKALNCONH<sub>2</sub>

In alternative embodiments of the peptides of the invention, underlined spacer monomers (such as the illustrated glycine G's) may for be used in variable numbers, such as 2, 3 or 4 glycines.

In alternative embodiments, internal cyclization of peptides of the invention may be in alternative positions, or between substituted amino acids. The nature of the cyclic linkage may also be varied. For example, the linkage may be shortened by replacing the relevant glutamate (E) with an aspartate (D) residue, and/or replacing the lysine (K) with an ornithine (O) residue. Cyclization is for example possible between Aspartic acid 24 (D24) and Lysine 20 or 28 (K20 or K28), as illustrated in some of the exemplified polypeptides.

10

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/D24-cyclic acid

H<sub>2</sub>NKPVSLSYRCPCRFFGGGGLKWIQDYLEKALNCOOH

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/D24-cyclic amide

H<sub>2</sub>NKPVSLSYRCPCRFFGGGGLKWIQDYLEKALNCONH<sub>2</sub>

15

Disulphide or sulphide bridging may be used to produce alternative embodiments of the polypeptides of the invention, in which cysteine residues may for example be involved in bridge formation, as indicated in the following sequences by double underlined residues.

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-C9/C11-cyclic acid

H<sub>2</sub>NKPVSLSYRCPCRFFGGGGLKWIQEYLEKALNCOOH

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-C9/C11-cyclic amide

H<sub>2</sub>NKPVSLSYRCPCRFFGGGGLKWIQEYLEKALNCONH<sub>2</sub>

In one aspect, polypeptide compounds of the invention may provide a CXCR4 agonist comprising a peptide having: (a) an N-terminal sequence homologous to a chemokine, such as an SDF-1 N-terminal sequence; (b) a C-terminal sequence homologous to a chemokine, such as an SDF-1 C-terminal sequence; (c) a linking moiety joining the N-terminal sequence to the C-

terminal sequence, such as a polypeptide linker; and, (d) an internal cyclic bridge formed between portions of the polypeptide, such as an amide linking a carboxylic acid side chain on a first amino acid residue and an amine side chain on a second amino acid residue. In some embodiments, the C-terminal sequence may comprise the internal cyclic bridge.

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As shown above, exemplary embodiments of polypeptides of the invention have been synthesized, having N-terminal SDF-1 residues (1-14) or (1-17), linked to C-terminal SDF-1 residues (55-67) by a three or four-glycine linker. In some embodiments, peptides are cyclized between glutamic acid (at 24 position) and lysine (at 20 or 28 position). Lactamization may be affected by removing the allylic group from both side chains of lysine and glutamic acid using the palladium technique and then effecting internal amide bond formation between the corresponding lysine and glutamic acid. Selected members of this family of polypeptides showed high affinity in a CXCR4 receptor binding assay (CEM cells) and in activating  $[Ca^{2+}]$  mobilization (THP-1 cells). Further embodiments of polypeptides are listed below:

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/D24-(E24 -> D)-cyclic acid or amide

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K28/D24-(E24 -> D)-cyclic acid or amide

Cyclization may also take place between ornithine (O) and glutamic acid (E):

20

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-O20/E24-(K20 ->O)-cyclic acid or amide

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-O28/E24-(K28 ->O)-cyclic acid or amide

Cyclization may also take place between ornithine (O) and aspartic acid (D):

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-O20/D24-(K20 -> O & E24 -> D)-cyclic acid or amide

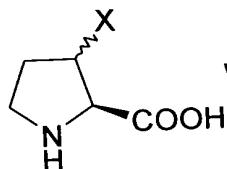
SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-O28/D24-(K28 -> O & E24 -> D)-cyclic acid

or amide

In some embodiments, proline (P) at position 6th may be replaced with serine (S). In some embodiments, lysine (K) and glutamic acid (E) may be replaced by ornithine (O) and aspartic acid (D), respectively. Similarly, substitutions may be made in the LSYR region, replacing lucine (L), serine (S), tyrosine (Y) or arginine (R) by proline (P) or other similarly shaped moiety. Alternatively, proline may be substituted with P\*:

10

Where P\* =

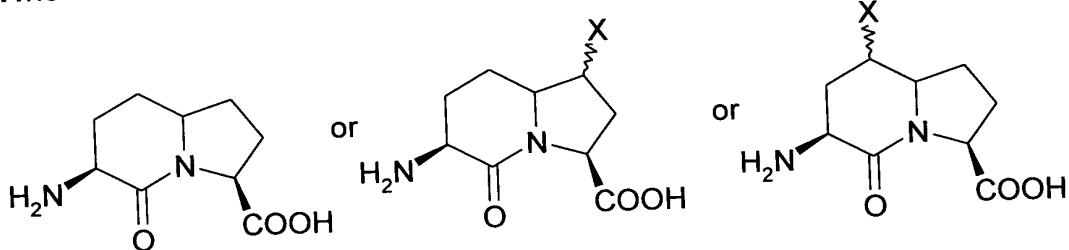


with X= Ar, Ar-OH, alkyl and more

A wide variety of amino acid substitutions may be made in polypeptide sequences, such as K to E, K to D, O to E, O to D. Moieties other than naturally occurring amino acids may also be substituted, such as Btd:

15

Where Btd\* =



X= Alkyl, Ar, Ar-OH and more

20

Similarly, polypeptides may be prepared using sequences from chemokines other than SDF-1. Such as residues 36-50, 10-50 or 55-70 of MIP-1 $\alpha$ :

SDF-1(1-14)-(G)<sub>4</sub>-MIP-1 $\alpha$ (36-50)-acid or amide

H<sub>2</sub>N-KPVSLSYRCPCCRFFGGGGSKPGVIFLT KRSRQV-CONH<sub>2</sub>

SDF-1(1-14)-(G)<sub>4</sub>-MIP-1 $\alpha$ (11-50)- acid or amide

H<sub>2</sub>N-

5 KPVSLSYRCPCCRFFGGGCCFSYTSRQIPQNFIA DYFETSSQCSKPGVIFLT  
KRSRQV-CONH<sub>2</sub>

SDF-1(1-14)-(G)<sub>4</sub>-MIP-1 $\alpha$ (56-70)-acid or amide

H<sub>2</sub>N-KPVSLSYRCPCCRFFGGGEEWWQKYVDDLELSA-CONH<sub>2</sub>

10

In various Figures, compounds are identified by abbreviations, as follows:

Structure of CTCE9901:

SDF-1 (1-9)<sub>2</sub>-C9/C9-cysteine dimer

H<sub>2</sub>NKPVSLSYRCCOOH

|

H2NKPVSLSYRCCOOH

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Structure of CTCE9902:

SDF-1(1-17)

H<sub>2</sub>NKPVSLSYRCPCCRFFFESHCOOH

25

Structure of CTCE9904:

SDF-1 (1-8)<sub>2</sub>-lysine bridge dimer

H<sub>2</sub>NKPVSLSYR

|

K-CONH<sub>2</sub>

|

H<sub>2</sub>NKPVSLSYR

35

Structure of CTCE0013:

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) acid

H<sub>2</sub>NKPVSLSYRCPCCRFFGGGLKWIQEYLEKALNCOOH

Structure of CTCE0017:

SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) amide

40

H<sub>2</sub>NKPVSLSYRCPCCRFFGGGLKWIQEYLEKALNCONH<sub>2</sub>

Structure of CTCE0022:  
SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide



5 Structure of CTCE0021:  
SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide



10 **Example 2**

Tables 1 and 2 show the effect of CXCR4 agonists on bone marrow progenitor cells, particularly primitive erythroide cells and primitive granulocytes, compared to mature granulocytes. To obtain the data in Tables 1 and 2, cells were pre-incubated with each of the compounds or saline alone ('no drug' as control). The cells were then exposed to high dose H<sup>3</sup>-thymidine, a cytotoxic agent. Rapidly dividing cells accumulate proportionally more of the cytotoxic radioactive thymidine and as a result are preferentially killed. The relative proportion of cells killed by the thymidine treatment compared to the control is indicative of the relative effectiveness of the compounds in reducing cellular multiplication, *i.e.* decreasing the rate of cell cycle progression. A higher (or unchanged) proportion of killed cells compared to the control is indicative that a compound does not reduce cellular multiplication of the given cell type.

25 **Table 1:**  
**Effect of CXCR4 Agonists on Bone Marrow Progenitor Cells Exposed to H<sup>3</sup>-Thymidine.**

|                       | % CELL KILLED     |       |                         |
|-----------------------|-------------------|-------|-------------------------|
|                       | No drug (control) | SDF-1 | SDF-1(1-9) <sub>2</sub> |
| Primitive Erythroide  | 71                | 2     | 9                       |
| Primitive Granulocyte | 46                | 1     | 1                       |
| Mature Granulocyte    | 39                | 45    | 42                      |

In Table 1, SDF-1 polypeptide (KPVSL SYRCP CRFFE SHVAR ANVKH LKILN TPNCA LQIVA RLKNN NRQVC IDPKL KWIQE YLEKA LN) is used at 100 ng/ml on a human bone marrow cell culture. SDF-1(1-9)<sub>2</sub> (KPVSLSYRC-X-CRYSLSPK) is used at 50 ug/ml on a human bone marrow cell culture.

5 Table 2 further demonstrates that SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-amide and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide are both able to inhibit cell cycling in human positive erythroid and primitive 10 granulopoietic cells, but not in mature granulopoietic cells, in the assay as described above in this Example.

**Table 2**

|                       | % CELL KILLED     |            |            |
|-----------------------|-------------------|------------|------------|
|                       | No drug (control) | Compound A | Compound B |
| Primitive Erythroide  | 47 +/- 4          | 5 +/- 3    | -7 +/- 6   |
| Primitive Granulocyte | 42 +/- 3          | 1 +/- 6    | -11 +/- 7  |
| Mature Granulocyte    | 48 +/- 3          | 39 +/- 5   | 44 +/- 6   |

15 Where: Compound A is SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-amide; Compounds B is SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide

### **Example 3**

The present example demonstrates the therapeutic effectiveness of 20 CXCR4 agonists in an animal model, showing protection of hematopoietic cells from cytotoxic treatments with CXCR4 agonists. In these animal studies, normal mice were treated with the cytotoxic chemotherapeutic agent arabinose-cytosine (Ara-C), which are known to deleteriously affect cells with high rates of DNA synthesis (reflecting rapid cell cycling).

As shown in the graph of Figure 1, in mice given a single dose of Arabinose Cytosine (Ara-C) at 350 mg/kg at day zero intravenously, white blood cell count (WBC) decreases (due to the cytotoxic action of Ara-C). In contrast, in mice given the peptide SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (designated CTC in the graph legend) in combination with Ara-C, the extent of white blood cell count decrease is significantly ameliorated. In the graph, circular data points correspond to the white blood cell count in animals that received Ara-C but did not receive the peptide, and triangular data points are for animals that received Ara-C and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide. The data clearly demonstrated the protective action of the peptide of the invention against the cytotoxic action of Ara-C.

#### Example 4

The efficacy of SDF-1 and SDF-1 peptide analogs as CXCR4 agonists was demonstrated through CXCR4 receptor binding assays. A competitive dose response for binding to the SDF-1 receptor by native SDF-1 and the CXCR4 agonists against <sup>125</sup>I-SDF-1 is shown in Figures 2A and 2 B respectively. A concentration-dependent inhibition of <sup>125</sup>I-SDF-1 is illustrated in Figure 2A, indicating the affinity of SDF-1 for the receptor. A Scatchard plot is illustrated, and the K<sub>D</sub> was determined to be 26nM. SDF-1 and the indicated analogs (competing ligands) were added at the concentrations illustrated in the presence of 4nM <sup>125</sup>I-SDF-1. CEM cells were assessed for <sup>125</sup>I-SDF-1 binding following 2 hr of incubation. The results are expressed as percentages of the maximal specific binding that was determined without competing ligand, and are the mean of three independent experiments. The inhibition of <sup>125</sup>I-SDF-1 by SDF-1 and the SDF-1 analogs is indicative of CXCR4 receptor binding. The compounds illustrated in the figure are as follows: SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021), SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide (CTCE0022), SDF-1(1-9)<sub>2</sub>-C9/C9-cysteine dimer (CTCE9901), SDF-1(1-17) (CTCE9902), SDF-1(1-8)<sub>2</sub>-lysine bridge dimer (CTCE9904) and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) amide (CTCE0017).

Example 5

This example illustrates the efficacy of SDF-1 and SDF-1 peptide analogs in mediating intracellular calcium mobilization ( $[Ca^{2+}]_i$ ). To illustrate that the binding of SDF-1 and SDF-1 peptide analogs results in the agonistic induction of the CXCR4 receptor,  $[Ca^{2+}]_i$  mobilization assays were conducted, the results of which are shown in Figure 3. To obtain the data shown in Figure 3, fura-2,AM loaded THP-1 cells ( $1 \times 10^6$ /ml) were stimulated with SDF-1, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) K20/E24-cyclic amide or SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide at the concentrations indicated (the values represent the mean +/- one S.D. of n=3 experiments). As shown by the data in Figure 3, incubation of THP-1 cells with SDF-1, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) K20/E24-cyclic amide or SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide resulted in the receptor-mediated induction of  $[Ca^{2+}]_i$  mobilization. The EC<sub>50</sub> values (the concentration of ligand necessary to effectively induce 50% of the full  $[Ca^{2+}]_i$  mobilization potential) for SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) acid, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide or SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide and native SDF-1 is shown in Table 3:

20

Table 3

| Compound  | EC <sub>50</sub> (nM) |
|---|-----------------------|
| SDF-1   | 26.56                 |
| SDF-1(1-14)-(G) <sub>4</sub> -SDF-1(55-67)-E24/K28-cyclic amide | 106.25                |
| SDF-1(1-14)-(G) <sub>4</sub> -SDF-1(55-67)-K20/E24-cyclic amide | 147.94                |
| SDF-1(1-14)-(G) <sub>4</sub> -SDF-1(55-67) acid                 | 188.30                |

25 The comparative ability of SDF-1, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021), SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-E24/K28-cyclic amide (CTCE0022), SDF-1 (1-9)<sub>2</sub>-C9/C9-cysteine dimer (CTCE9901), SDF-1(1-17) (CTCE9902), SDF-1 (1-8)<sub>2</sub>-lysine bridge dimer (CTCE9904) and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) amide (CTCE0017) to induce  $[Ca^{2+}]_i$  mobilization at the ligand concentration that the native SDF-1

gave maximal  $[Ca^{2+}]_i$  mobilization ( $1\mu M$ , refer to Figure 3) is illustrated in Figure 4. Fura-2,AM loaded THP-1 cells ( $1 \times 10^6/ml$ ) were stimulated with native SDF-1 and the SDF-1 peptide agonist analogs at the concentration of native SDF-1 that gave the maximum  $[Ca^{2+}]_i$  stimulation ( $1\mu M$ ) (the values represent the mean +/- one S.D. of  $n=3$  experiments).

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### Example 6

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Primitive high proliferative potential colony forming cells (HHP-CFC) in an adherent layer in culture are usually in a quiescent state. This long term culture (LTC) is established seven to ten days after initiation of the LTC. The cells may be stimulated to proliferate by the addition of fresh medium. Both BFU-E (burst forming unit – erythroid precursor) cells and CFU-GM (colony forming unit – granulocyte-monocyte common precursor) cells of LTC may be maintained in a quiescent state by the mesenchymally derived stromal cells in an adherent layer, but can be reversibly stimulated into the cycle by the addition of fresh media. The ability of CXCR4 agonists such as SDF-1 and SDF-1 polypeptides to overcome this activation may be determined by adding it to the LTC during the medium change. Rapidly dividing cells will accumulate proportionally more of a cytotoxic agent, such as radioactive thymidine, and as a result are preferentially killed.

25

The results depicted in Table 4 illustrate the ability of SDF-1, and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021) and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) acid (CTCE0013) to repress the proliferation of clonogenic erythroid and granulopoietic progenitors (which differentiate into erythrocytes, platelets, monocytes/macrophages and neutrophils) in an *in vitro* LTC-IC (long-term culture-initiating cells) assay.

30

**Table 4. Effect of SDF-1 and SDF-1 agonists on the cycling of primitive progenitors in the adherent layer of human LTC.**

% Kill after  $^3H$ -Thymidine

| <u>Treatment</u> | <u>Dose</u>     | <u>Primitive BFU-E</u>        | <u>Primitive CFU-GM</u> |
|------------------|-----------------|-------------------------------|-------------------------|
| <b>None</b>      |                 | 48 +/- 4                      | 44 +/- 3                |
| 5                | <b>CTCE0013</b> | 1 $\mu$ g/ml<br>10 $\mu$ g/ml | 24 +/- 6<br>0 +/- 2     |
|                  | <b>SDF-1</b>    | 1 $\mu$ g/ml                  | 4 +/- 3                 |
| 10               | <b>CTCE0021</b> | 1 $\mu$ g/ml                  | 2 +/- 4                 |
|                  |                 |                               | 0 +/- 3                 |

To obtain the results set out in Table 4, clonogenic erythroid (BFU-E) and granulopoietic (CFU-GM) progenitors were assayed in methylcellulose cultures. Adherent cells were treated with fresh medium alone (as control) or 15 with the indicated CXCR4 agonist (10  $\mu$ g/ml SDF-1, CTCE0021 or CTCE0013). Dishes were harvested three days later and  $^3$ H-thymidine suicide assays performed on the progenitor cells in the adherent layer to determine the proportion of cells killed as a result of accumulation of cytotoxic  $^3$ H-thymidine, where the difference between the cells in the control and the 20 number of cells remaining represent the cells killed.

Figure 5 illustrates that feeding cultures SDF-1 in conjunction with media changes results in significantly reduced cell mortality of hematopoietic cells when the cells are challenged with an agent that is preferentially cytotoxic to dividing cells, in which circles represent BFU-E cells (burst forming unit-erythroid precursors), and squares represent CFU-GM cells (colony forming unit-granulocyte-monocyte common precursor). Figure 6 shows that a similar concentration dependent effect may be obtained with 25 SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/D24-cyclic amide (Compound #1) and SDF-1(1-9)<sub>2</sub> (Compound #3). Together, Figures 5 and 6 illustrate that the 30 SDF-1 polypeptide and SDF-1 peptide analogs repress the cyclic activation of the BFU-E and CFU-GM progenitor stem cells in the adherent layer of LTC.

Example 7

Figures 7 and 9 show the efficacy of CXCR4 agonists such as SDF-1 and SDF-1 analogues in repressing the proliferation of human progenitor cells in an *in vivo* engraftment model.

5 In Figure 7, the cycling status of mature and primitive colony forming cells (CFU-GM; colony forming unit-granulocyte-monocyte precursor, BFU-E; burst forming unit-erythroid precursor; LTC-IC, long-term culture initiating cell) in the suspension of CD34<sup>+</sup> cells isolated from the marrow of transplanted  
10 NOD/SCID mice was determined by assessing the proportion of these progenitors that were inactivated (killed) by short term (20 min) or overnight (16 hour) exposure of the cells to 20 $\mu$ g/ml of high specific activity <sup>3</sup>H-thymidine (values represent the mean +/- the S.D. of data from up to four experiments with up to four mice per point in each). Significant in the results  
15 described in Figure 4 is the observation that the analogs SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021) and SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) acid (CTCE013) are as effective as native SDF-1 at inhibiting the proliferation of "primitive" human progenitor cells, as measured by the reduction of cells killed by exposure to high specific activity <sup>3</sup>H-thymidine  
20 (which only affects proliferating cells).

Example 8

SDF-1 enhances the delectability of colony regenerating units (CRU) regenerated in NOD/SCID mice transplanted with human fetal liver cells (Figure 8). Three to four NOD/SCID mice per group were sublethally irradiated and injected with human cells, in this case 10<sup>7</sup> light density fetal liver cells, and the mice then maintained for an interval of 2.5-3 weeks. As indicated, each group was then given 2 daily injections of either 10 $\mu$ g of SDF-1, or an equivalent volume of control medium, and all mice were then sacrificed one day after the second injection. The bone marrow cells from each group were then pooled, and an aliquot removed for FACS analysis and overnight <sup>3</sup>H-thymidine suicide assays to measure the cycling activity of the

human CFC and LTC-IC (long term culture initiating culture) present. The remainder of the cells were injected into groups of 3-6 secondary recipients. These animals were then sacrificed 6 to 8 weeks later and their bone marrow removed and analysed for the presence of human cells.

5

This example describes a secondary engraftment. When the bone marrow of the secondary recipients was evaluated, a considerable difference was observed in the level of human cells present in recipients of cells from the different groups of primary mice. As shown in Figure 8, for SDF-1-injected 10 mice a far greater number of all types of human cells assessed was found in the marrow of the secondary recipients that had received marrow from primary mice treated with either SDF-1 by comparison to recipients of cells from media injected control primary mice.

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### **Example 9**

This example illustrates the effect of CXCR4 agonists such as SDF-1 and SDF-1 polypeptide analogs on the engraftment of human cells in human fetal liver transplanted NOD/SCID mice (Figure 9). As shown in this figure, there was a lack of short-term effect of CXCR4 agonists on the frequency of 20 different human cells present in NOD/SCID mice. In these experiments, 6 to 8 weeks post-transplanted mice were injected two times, one day apart with the test compound (SDF-1, SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021) or SDF-1(1-14)-(G)<sub>4</sub>-SDF-1(55-67) acid (CTCE013)) and sacrificed one day later. The frequency of the phenotypically defined human 25 hematopoietic cells detected in the long bones (tibias and femurs) of mice was determined. Administration of 0.5mg/kg of SDF-1 had no significant effect on the number of CD45/71, CD19/20, or CD34 cells, nor on the CFC or LTC-IC. In addition, none of the human cell types were detectably affected by this 30 schedule of CXCR4 agonist administration. This data, coupled with that of Figures 7 and 8, indicates that SDF-1, SDF-1 analogs and other CXCR4 agonists may effectively augment secondary engraftment of human progenitor cells.

Example 10

This example illustrates the effect of an SDF-1 polypeptide analog CTCE0021 (10mg/kg, identified as Compound #1 in Figure 12) on the recovery of leukocytes following myeloablative chemotherapy with Ara-C (300mg/kg). In the experiment described in the example, C3H<sup>hen</sup> mice (female) were treated with 500mg/kg Ara-C for two cycles - on days 0 and 10. During the second cycle of Ara-C dosing, Ara-C treated mice were injected with 10mg/kg CTCE0021 each day. A control was conducted with animals treated with Ara-C alone. Blood was collected from the tail vein into heparin-containing tubes at the onset of the experiment, and one day before every day following the second Ara-C dose. A total leukocyte count was determined. As shown in the graph of Figure 10, the CXCR4 agonist CTCE0021 acted to inhibit the cytotoxic effects of Ara-C and to sustain a higher level of leukocytes, illustrating the reversal of myelosuppressive effects of a chemotherapeutic regimen *in vivo*.

Example 11:

This example illustrates the effect of an SDF-1 polypeptide analog SDF-1(1-14)-(G)4-SDF-1(55-67)-K20/E24-cyclic amide (CTCE0021, 1mg/kg) on the recovery of leukocytes following myeloablative chemotherapy with Ara-C (500mg/kg) compared to G-CSF (Neupogen<sup>®</sup>) (Figure 11). C3H<sup>hen</sup> mice (female) were treated with 500mg/kg Ara-C for two cycles - on days 0 and 10. During the second cycle of Ara-C dosing, Ara-C treated mice were injected with 10mg/kg CTCE0021, 10mg/kg Neupogen<sup>®</sup>, alone or together (on days - 1, 0, and 1 to 3), with controls receiving no drug. Blood was collected from the tail vein into heparin-containing tubes at the onset of the experiment, and one day before and 1, 7 and 12 days following the second Ara-C dose. A total white blood cell count was obtained. The results in this example indicates that not only does treatment with CTCE0021 enhance the recovery of white blood cells following myeloablative chemotherapy with Ara-C, co-treatment with the SDF-1 polypeptide analog and G-CSF (Neupogen<sup>®</sup>)

resulted in a greater recovery compared the animals treated with G-CSF alone during the early treatment phase. Furthermore, the recovery following treatment with the SDF-1 polypeptide analog was sustained compared to the G-CSF treated animals.

5

Figure 12 depicts the results of an experiment conducted under identical conditions, but the growth (increase in leukocyte count) relative to the number of cells counted in animals treated with Ara-C alone is illustrated. By twelve days following Ara-C administration, an approximately 7.5-fold increase in leukocytes was observed in mice treated with CTCE0021 relative to animals treated with Ara-C alone, compared to 180% obtained in animals treated with Neupogen®.

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